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Molecular identification of a component of delayed rectifier current in gastrointestinal smooth muscles



FELICITAS SCHMALZ, JACQUELINE KINSELLA, SANG DON KOH, FIVOS VOGALIS, ANNE SCHNEIDER, ELAINE R. M. FLYNN, JAMES L. KENYON, AND BURTON HOROWITZ
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Schmalz, Felicitas, Jacqueline Kinsella, Sang Don Koh, Fivos Vogalis, Anne Schneider, Elaine R. M. Flynn, James L. Kenyon, and Burton Horowitz. Molecular identification of a component of delayed rectifier current in gastrointestinal smooth muscles. *Am. J. Physiol.* 274 (*Gastrointest. Liver Physiol.* 37): G901–G911, 1998. — Kv2.2, homologous to the *shab* family of *Drosophila* voltage-gated K⁺ channels, was isolated from human and canine colonic circular smooth muscle-derived mRNA. Northern hybridization analysis performed on RNA prepared from tissues and RT-PCR performed on RNA isolated from dispersed and selected smooth muscle cells demonstrate that Kv2.2 is expressed in smooth muscle cells found in all regions of the canine gastrointestinal (GI) tract and in several vascular tissues. Injection of Kv2.2 mRNA into *Xenopus* oocytes resulted in the expression of a slowly activating K⁺ current (time to half maximum current, 97 ± 8.6 ms) mediated by 15 pS (symmetrical K⁺) single channels. The current was inhibited by tetraethylammonium (IC₅₀ = 2.6 mM), 4-aminopyridine (IC₅₀ = 1.5 mM at +20 mV), and quinine (IC₅₀ = 13.7 μ M) and was insensitive to charybdotoxin. Low concentrations of quinine (1 μ M) were used to preferentially block the slow component of the delayed rectifier current in native colonic myocytes. These data suggest that Kv2.2 may contribute to this current in native GI smooth muscle cells.

colon; potassium channel; cloning; cDNA

K⁺ CHANNELS OF smooth muscle plasma membranes regulate contraction by setting the membrane potential, thus controlling the influx of Ca²⁺ through voltage-gated Ca²⁺ channels. Voltage-clamp studies of isolated myocytes have characterized K⁺ currents and identified two types of outwardly rectifying K⁺ channels: voltage-gated "delayed rectifier" channels that are insensitive to intracellular Ca²⁺, along with Ca²⁺- and voltage-activated channels (e.g., large-conductance Ca²⁺-activated K⁺ channels). Furthermore, the delayed rectifier current in canine colonic smooth muscle consists of three current components with characteristic voltage dependencies, kinetics, and pharmacology (4). In particular, $I_{dK(f)}$ is a fast-activating current blocked by micromolar concentrations of 4-aminopyridine (4-AP), $I_{dK(s)}$ is a slowly activating current blocked by tetraethylammonium (TEA), and $I_{dK(n)}$ is a TEA-sensitive current that inactivates at negative potentials.

Although some of the functions of these channel types have been elucidated by pharmacological and physiological studies of intact smooth muscle cells (2, 10, 12, 34), much important information about their roles in the control of smooth muscle function remains to be revealed. This is due in part to difficulties interpreting data from preparations expressing several

different kinds of K⁺ channels. The difficulty is compounded when the dissection is approached at the single-channel level (18). An alternate approach to these questions is the application of molecular biological techniques that identify genes encoding different K⁺ channels and that produce cells expressing only one kind of K⁺ channel for electrophysiological study. We (14, 22) have used this approach and probed mRNA isolated from gastrointestinal (GI) and vascular smooth muscles with degenerate oligonucleotide primers designed to hybridize to conserved sequences surrounding the pore region of members of the Kv1–4 family of K⁺ channels. This has led to the cloning and expression of two delayed rectifier K⁺ channels [Kv1.2 (14) and Kv1.5 (22)]. Heterotetramers of the proteins encoded by these genes probably underlie $I_{dK(f)}$ in canine colonic smooth muscle (27).

In the present study, we describe the identification of transcripts encoding a Kv2.2 channel in myocytes from several smooth muscles. This family, related to the *shab* channels in *Drosophila*, was first characterized in mammalian brain by Hwang et al. (16), who found that Kv2.1 and Kv2.2 transcripts were differentially localized in the central nervous system and that currents mediated by Kv2.2 activated relatively slowly and were inhibited by TEA. Slow activation is apparently a unique feature of Kv2 channels, as it has been observed in Kv2.1 (11) but not in other Kv families. In contrast, the sensitivity of the Kv2.1 channels to 4-AP varies among the members of the family: Frech et al. (11) found a high sensitivity, whereas Pak et al. (24) found that their channels were virtually insensitive to this agent. We describe the electrophysiological and pharmacological properties of Kv2.2 cloned from human and canine colonic smooth muscle. We also determine the transcriptional expression pattern of Kv2.2 and a β -subunit (β_4) found to couple to Kv2.2 (9), in isolated myocytes of several GI, uterine, and vascular smooth muscles utilizing RT-PCR on mRNA prepared from isolated myocytes and Northern hybridization on mRNA prepared from tissue. We suggest that Kv2.2 may underlie a component of delayed rectifier current in many smooth muscles.

METHODS

Tissue dissection and mRNA preparation. Canine colonic circular and longitudinal muscles were dissected as previously described (3). Samples of human sigmoid colon were obtained from 23 volunteer patients during elective colon resections for nonobstructive neoplasms and provided to us by Byron McGregor (Department of Surgery, University of Nevada). Circular smooth muscle was prepared from human tissue as described previously (17). GI and other smooth

muscle tissues were dissected free of fat and connective tissue under a dissecting microscope. Poly(A)⁺ RNA was prepared from dissected tissue using the Fast Track kit (Invitrogen, San Diego, CA), according to the manufacturer's instructions. Muscles from four to five animals (0.3–0.5 g) were pooled for each RNA preparation. Each sample of RNA was adjusted to the same concentration. Poly(A)⁺ RNA was also isolated from canine brain using this method.

Total RNA preparation from isolated myocytes. Elongated smooth muscle cells were identified under magnification using the same criteria as that used for electrophysiological examination. These cells (50–60) were drawn up into a capillary pipette and expelled into a 0.5-ml tube. Only spindle-shaped cells (length 20–500 μ m; diameter 5–60 μ m) were selected. The cells were snap frozen in liquid nitrogen and stored at -70°C until RNA preparation. RNA was prepared from isolated cells using a modification of the guanidinium thiocyanate procedure (7). Polyinosinic acid (20 μ g) was added as a carrier (35). Myocytes from the circular and longitudinal muscle layers of tissues from several regions of the GI tract and other smooth muscles were prepared, as previously described (5).

cDNA isolation and nucleotide sequencing. Canine Kv2.2 (cKv2.2) and human Kv2.2 (hKv2.2) cDNAs were isolated with a modified RT-PCR method (30). First-strand cDNA was prepared from human and canine colonic circular smooth muscle-derived mRNA in a 60- μ l reaction containing 100 ng of oligo(dT) primers or random oligonucleotides, 12 μ l of 5 \times first-strand buffer (GIBCO-BRL, Gaithersburg, MD), 6 μ l of 0.1 M dithiothreitol, 15 μ l of 5 mM dNTPs (Invitrogen), 200 U of Moloney murine leukemia virus RT (GIBCO-BRL), 40 U of RNase inhibitor (Promega, Madison, WI), and 300 ng of poly(A)⁺ RNA. The reaction was incubated at room temperature for 10 min and at 42°C for 50 min, then heated to 70°C for 15 min, and cooled on ice for 10 min. We added 20 U of RNase H (Promega), and the reaction was further incubated for 20 min.

PCR was performed utilizing several overlapping primer oligonucleotides. Initially, degenerate primers designed to amplify the conserved pore region for the mammalian Kv2 family were used to determine if any Kv2 homologue was expressed in colonic myocytes. Once an amplification product corresponding to the Kv2.2 pore region was identified, oligo(dT) and specific primers designed to hybridize to sequence in the 3' untranslated region were used as 3' anchor-reverse primers in conjunction with several primers (in individual reactions) encoding regions of the rat Kv2.2 (rKv2.2) sequence (16) for the 5' forward primer. Nucleotide positions (nt) are relative to the cKv2.2 coding sequence. The primers used were as follows: *primer 1*, (pore forward) 5'-CTC-CA(A,C)-GA(A,G)TCCAACAA(A,G)AG(C,T)GTGC-3' (nt 847–871); *primer 2*, (pore reverse) 5'-TTCAT(A,G)GA(A,C,T)AC-GATGCT(G,T)CC(A,G)-3' (nt 1338–1358); *primer 3*, 5'-CCATGGCAGAAAAGGCACCTCCTGG-3' (nt –2–23); *primer 4*, 5'-GGGAAGCTCCGAGACTGTAACACGC-3' (nt 184–208); *primer 5*, 5'-CCCACTCATTTGTAACCTCGCC-3' (nt 985–1007); and *primer 6*, 5'-CCAGTGTCTATTTTCAGTGAAG-CTCC-3' (nt 2393–2417). In the 5' region of rKv2.2 sequence, an anchor primer based on the sequence just upstream from the start codon was utilized (*primer 3*). This primer was used in conjunction with *primers 2* and *5* (in individual reactions) encoding conserved regions of Kv2.2 structure for the 3' reverse primer. In the 3' region *primer 5* was used as the anchor primer and paired with *primers 1*, *3*, and *4*.

PCR was performed in a reaction volume of 50 μ l in buffer containing 2.3 mM MgCl_2 , 0.1 mM of each dNTP, 0.2 μ M of each primer, and 0.5 U of *Taq* polymerase (Promega). The

reaction was performed with an initial denaturation of 5 min followed by 33 cycles (94°C for 30 s; 55°C for 30 s; 72°C for 20 s) and a final extension step of 10 min at 72°C in a thermal cycler (Coy). Reaction products (3 μ l) were electrophoresed through a 1% agarose-40 mM Tris acetate, 2 mM EDTA gel and visualized with ethidium bromide.

Amplification products were subcloned into the PCR II vector (Invitrogen) using direct ligation in a 10- μ l reaction containing 2 μ l of the amplification products, 1 μ l (10 ng) T/A vector DNA, 1 μ l 10 \times ligation buffer (Invitrogen), and 4 U of T4 ligase (Invitrogen). The ligation reaction was transformed into competent *Escherichia coli* and spread onto ampicillin plates. White colonies were picked (blue/white selection) and analyzed for amplification product inserts. Subclones were analyzed by nucleotide sequencing. Several were analyzed for each product subcloned, and overlapping regions were examined for sequencing accuracy. To determine transcriptional expression from isolated cells derived from GI, vascular, and uterine smooth muscles, a primer pair was designed from the deduced cKv2.2 nucleotide sequence. The region is specific for Kv2.2 and corresponds to nt 184–1007 (*primers 4* and *5*), generating an 823-nt amplification product. Expression of the Kv2.2 β -subunit β_4 (9) was determined by RT-PCR analysis using primers designed from the β_4 sequence to be specific for this cDNA (forward, ATGTCAAGAGGGTATGGTCTGATAT, nt 1–24 of the coding sequence; reverse, CCAAGAGCGCATC-TATTTCCACCAC, nt 701–724 of the coding sequence). Expression of Kv8.1 was determined by RT-PCR analysis using primers designed from the Kv8.1 sequence to be specific for this cDNA (15) (forward, TCAACGTGGGCGGCAGCCGCT-TCGT, nt 290–317 of the coding sequence; reverse, CCTTCT-TAAAGTCCAGCGTTTCGCT, nt 646–670 of the coding sequence).

For expression of the complete Kv2.2 open reading frame (ORF), two clones that overlapped by 500 bp were joined by ligation at a common *Bam*H I site. This construct was subcloned into a *Xenopus* oocyte expression vector (pSP64T) (19) (kindly provided by D. Melton, Harvard University, Cambridge, MA) downstream from the SP6 promoter. The plasmid was linearized with *Kpn* I, and capped transcripts were synthesized in vitro with SP6 RNA polymerase, as described previously (19). Transcripts were resuspended in 10 mM Tris (pH 7.4), 1 mM EDTA at a final concentration of 1 μ g/ μ l.

Northern analysis. RNA was size fractionated on 1.0% agarose-formaldehyde gels and transferred to Immobilon filters (33). Filters were baked and prehybridized in 50% formamide, 5 \times SSC (1 \times SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 50 mM sodium phosphate, 5 \times Denhardt's solution, sonicated salmon sperm DNA (50 μ g/ml), 0.1% SDS, and 10% dextran sulfate at 42°C overnight. Restriction endonuclease fragment (*Bgl* II) specific for Kv2.2 was prepared from the clone (nt 1–389), and the probe was labeled with ^{32}P by using a random-primer technique (8). Hybridization was performed under the same conditions overnight. The filters were washed at high stringency (3 times in 2 \times SSC at room temperature for 5 min each, then twice for 30 min each in 0.2 \times SSC, 0.1% SDS at 65°C) to ensure specificity of labeling. Specific for a Chinese hamster ovary (CHO) cell mRNA expressed at equivalent levels in all tissue examined (13), a cDNA of CHO-B was used as an internal standard to verify that equal amounts of poly(A)⁺ RNA were applied. After hybridization, filters were exposed to intensifying screens and autoradiography was performed using a Bio-Rad Phosphorimager (Hercules, CA).

Oocyte injection and electrophysiological methods. Ovarian lobes were removed from anesthetized adult female *Xenopus*

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Xenopus laevis frogs (Xenopus 1, Ann Arbor, MI) under sterile conditions. The lobes were then mechanically opened, and the oocyte follicular layer was removed by incubation with collagenase (1 mg/ml) in Ca^{2+} -free ND96 (see below) solution at room temperature for 2–3 h. The oocytes were then collected, rinsed, and stored in ND96 solution containing (in mM) 2.5 pyruvate, 96 NaCl, 1.5 CaCl_2 , 2 KCl, 1 MgCl_2 , and 5 HEPES plus antibiotics (100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin) at 19°C for up to 24 h before injection. Only stage V and VI oocytes were selected for injection. mRNA (1 $\mu\text{g}/\mu\text{l}$) was injected in a total volume of 50 nl, and the oocytes were stored for 2–4 days until assay.

Whole cell K^+ currents were recorded using the double-electrode voltage-clamp technique (GeneClamp 500, Axon Instruments, Foster City, CA). Microelectrodes were filled with 3 M KCl and had resistances of 1–3 M Ω . Recordings were made at room temperature in a solution containing (in mM) 96 NaCl, 2 KCl, 2.8 MgCl_2 , and 5 HEPES, pH 7.4. Ca^{2+} was omitted from the solution to minimize the endogenous Ca^{2+} -activated chloride currents. Single K^+ channels were recorded in inside-out patches obtained from oocytes after mechanical removal of the vitelline membrane as described by Methfessel et al. (20). Currents were recorded by an Axopatch-1D amplifier (Axon Instruments) and filtered at 500 Hz before digitization. Patch pipettes were filled with (in mM) 140 KCl, 0.1 GdCl_3 , and 10 HEPES, pH 7.2. The bath solution contained (in mM) 140 KCl, 0.1 GdCl_3 , 10 HEPES, and 1 EGTA, pH 7.2. To establish the selectivity of the channels, 140 mM KCl was replaced with NaCl in some experiments. GdCl_3 was included to inhibit stretch-activated channels. In all voltage-clamp experiments, data acquisition and analyses were done using pCLAMP software (Axon Instruments). Data are expressed as means \pm SE.

Native colonic myocytes were dispersed and patch-clamp recordings performed as previously described (4). Bath solution contained (in mM) 140 NaCl, 5 KCl, 2 MnCl_2 , 1.2 MgCl_2 , 10 dextrose, 10 HEPES, and 5 Tris, pH 7.4. Pipette solution contained (in mM) 20 KCl, 110 potassium gluconate, 5 MgCl_2 , 2.5 K_2ATP , 0.1 Na_2GTP , 2.5 disodium creatine phosphate, 5 HEPES, and 1 1,2-bis(2-aminophenoxy)ethane- N,N,N',N' -tetraacetic acid (pH 7.2).

In some experiments, 4-AP (5 mM), TEA (10 mM), and quinine (1 μM) were added to the bath.

RESULTS

cDNA cloning and DNA sequence analysis. RT-PCR utilizing primers designed to generate several overlapping amplification products was used to construct the full ORF for the canine and human homologues of Kv2.2 cDNA. Several primer combinations resulted in amplification products overlapping in both the 5' and 3' orientations (see METHODS). Amplification products were separated on agarose gels and analyzed specifically for products of an unexpected size, which might indicate alternative splicing or homologous isoforms. Gels were also blotted and hybridized to previously isolated Kv2.2-specific amplification products as probes to detect low-level transcriptional expression of alternated forms of Kv2.2. None were detected for these amplifications. At least five individual subclones were completely sequenced for each of the overlapping products to ensure the fidelity of the RT-PCR reaction. We constructed a full-length ORF using two amplification products overlapping by 823 nt (3' primers 5 and 6; 5' primers 3 and 4). The complete amino acid sequence of the cKv2.2 and

hKv2.2 clones is shown in Fig. 1. The sequence is aligned with the translated rat brain cDNA sequence (16). Nucleotide sequence homology and amino acid sequence identity of cKv2.2 is 84.9% and 91.7% with hKv2.2 and 82.9% and 88.3% with rKv2.2. The majority of sequence divergence occurs in the carboxy-terminal domain.

Northern hybridization of cKv2.2 in visceral and vascular smooth muscles. Poly(A)⁺ RNA was prepared from several dissected regions of the canine GI tract. Longitudinal and circular muscle layers were separated. Smooth muscle tissue from coronary artery (>300- μm -diameter vessels) was also analyzed to determine the distribution of this smooth muscle-derived K^+ channel in a vascular smooth muscle. Canine brain tissue was also included to compare expression in the tissue source used for the initial cloning of Kv2.2 (16). A cDNA of CHO-B, specific for a CHO cell mRNA expressed at equivalent levels in all tissues examined (13), was used as an internal standard to verify that equal amounts of poly(A)⁺ RNA were applied. A specific probe for Kv2.2 was used for the hybridization. Figure 2A displays the autoradiogram resulting from the hybridization.

cKv2.2 hybridized to a 13-kb transcript in all canine tissues in which expression was detected. An additional transcript of ~6 kb hybridized in several tissues and was prominent in canine colon circular smooth muscle.

RT-PCR analysis of transcriptional expression of Kv2.2 in canine smooth muscle cells. To eliminate the possibility that detection of transcriptional expression of Kv2.2 was due to expression in a minor cell population in the smooth muscle (i.e., nerve, endothelial, interstitial cells), we prepared RNA from isolated smooth muscle cells. Myocytes were dispersed from several canine smooth muscles, including several regions of the GI tract, uterus, and vascular muscles. Fifty to sixty cells were identified based on criteria previously used for selection of cells for electrophysiological studies (4). Only spindle-shaped cells (length 20–500 μm ; diameter 5–60 μm) were selected. Total RNA was prepared from the selected cells, and specific primers, designed to amplify an 823-nt product of cKv2.2, were used in the RT-PCR reaction. Although colonic longitudinal muscle displayed relatively little amplification product, product was detectable on longer photographic exposure. The Kv2.2 β_4 auxiliary subunit expression was determined by using oligonucleotides specific for this cDNA and designed to amplify a 724-nt product. Expression of both Kv2.2 and β_4 was detected in cells from all of the smooth muscles tested (Fig. 2B). In contrast, Kv8.1, a Kv channel found to be associated with Kv2.2 in neuronal tissues (15), was not detected in any smooth muscle tested. The primers do amplify the appropriately sized product from canine brain (data not shown). PCR performed on cDNA from an RT reaction in which no RT was included did not generate any amplification product. This negative control tests whether DNA contaminates the RNA prepared from the isolated cells. We did not perform quantitative PCR on these samples and cannot report on the relative

cKv2.2	1	MAEKAPPGLN	RKTSRSTLSL	PPEPVDIIRS	KTCSRVRKIN	VGGLNHEVLW	50
hKv2.2	1	MAEKAPPGLN	RKTSRSTLSL	PPEPVDIIRS	KTCSRVRKIN	VGGLNHEVLW	50
rKv2.2	1	MAEKAPPGLN	RKTSRSTLSL	PPEPVDIIRS	KTCSRVRKIN	VGGLNHEVLW	50
cKv2.2	51	RTLDRLPRT	LGKLRDCNTH	ESLLEVCCDY	NLGHNEYFFD	RHPGAFTSIL	100
hKv2.2	51	RTLDRLPRT	LGKLRDCNTH	ESLLEVCCDY	NLGHNEYFFD	RHPGAFTSIL	100
rKv2.2	51	RTLDRLPRT	LGKLRDCNTH	ESLLEVCCDY	NLGHNEYFFD	RHPGAFTSIL	100
cKv2.2	101	NFYRTGKLHM	MEEMCALSG	QELDYWGIDE	IYLESCCQAR	YHQQKEQMNE	150
hKv2.2	101	NFYRTGKLHM	MEEMCALSG	QELDYWGIDE	IYLESCCQAR	YHQQKEQMNE	150
rKv2.2	101	NFYRTGKLHM	MEEMCALSG	QELDYWGIDE	IYLESCCQAR	YHQQKEQMNE	150
S1							
cKv2.2	151	ELRREAETMR	DGEGEEDFNT	CCPEKRRKSR	DLLEKPNSSV	AAKILAIVSN	200
hKv2.2	151	ELRREAETMR	DGEGEEDFNT	CCPEKRRKSR	DLLEKPNSSV	AAKILAIVSN	200
rKv2.2	151	ELRREAETMR	DGEGEEDFNT	CCPEKRRKSR	DLLEKPNSSV	AAKILAIVSN	200
S2							
cKv2.2	201	LFIVLSTIAL	SLNTLPELQE	MDEFGQPNND	ROLAHVEAVC	IAWFTMEYLL	250
hKv2.2	201	LFIVLSTIAL	SLNTLPELQE	MDEFGQPNND	ROLAHVEAVC	IAWFTMEYLL	250
rKv2.2	201	LFIVLSTIAL	SLNTLPELQE	MDEFGQPNND	ROLAHVEAVC	IAWFTMEYLL	250
S3							
cKv2.2	251	RFLSSPNKWK	FFKGPLNVID	LLAILPYVVT	IFLTESNKS	V LQFQNVRRV	300
hKv2.2	251	RFLSSPNKWK	FFKGPLNVID	LLAILPYVVT	IFLTESNKS	V LQFQNVRRV	300
rKv2.2	251	RFLSSPNKWK	FFKGPLNVID	LLAILPYVVT	IFLTESNKS	V LQFQNVRRV	300
S4							
cKv2.2	301	QIFRIMRILR	ILKLARHSTG	LQSLGFTLRR	SYNELGLLIL	FLANGIMIFS	350
hKv2.2	301	QIFRIMRILR	ILKLARHSTG	LQSLGFTLRR	SYNELGLLIL	FLANGIMIFS	350
rKv2.2	301	QIFRIMRILR	ILKLARHSTG	LQSLGFTLRR	SYNELGLLIL	FLANGIMIFS	350
H5-PORE							
cKv2.2	351	SLVFFAEKDA	DATKFTSIPA	SEFWATITMT	TVGYGDIYPK	TLLCKSVGG	400
hKv2.2	351	SLVFFAEKDE	DATKFTSIPA	SEFWATITMT	TVGYGDIYPK	TLLCKSVGG	400
rKv2.2	351	SLVFFAEKDE	DATKFTSIPA	SEFWATITMT	TVGYGDIYPK	TLLCKSVGG	400
S6							
cKv2.2	401	CCTAGVLVIA	LPIPIIVNPF	SEFYKEQKRO	EKAIKRREAL	ERAKRNGSIV	450
hKv2.2	401	CCTAGVLVIA	LPIPIIVNPF	SEFYKEQKRO	EKAIKRREAL	ERAKRNGSIV	450
rKv2.2	401	CCTAGVLVIA	LPIPIIVNPF	SEFYKEQKRO	EKAIKRREAL	ERAKRNGSIV	450
cKv2.2	451	SMNLKDFAFR	SMELIDVAVE	KAGESSTK	SADDNHLSPS	RWKWARKALS	500
hKv2.2	451	SMNLKDFAFR	SMELIDVAVE	KAGESSTK	SADDNHLSPS	RWKWARKALS	500
rKv2.2	451	SMNLKDFAFR	SMELIDVAVE	KAGESSTK	SADDNHLSPS	RWKWARKALS	500
cKv2.2	501	ETSSNKSYEN	KYQEVSKQDS	HEQLNNTSS	SPQHLSAQKL	EMLYNEITKT	550
hKv2.2	501	ETSSNKSYEN	KYQEVSKQDS	HEQLNNTSS	SPQHLSAQKL	EMLYNEITKT	550
rKv2.2	501	ETSSNKSYEN	KYQEVSKQDS	HEQLNNTSS	SPQHLSAQKL	EMLYNEITKT	550
cKv2.2	551	QPHFAPNPDG	QEQPDRPSAY	EEEIEMEEVV	CPQEQLAVAL	GEVIVDMKST	600
hKv2.2	551	QPHFAPNPDG	QEQPDRPSAY	EEEIEMEEVV	CPQEQLAVAL	GEVIVDMKST	600
rKv2.2	551	QPHFAPNPDG	QEQPDRPSAY	EEEIEMEEVV	CPQEQLAVAL	GEVIVDMKST	600
cKv2.2	601	SSIDSFTSCA	TDFETETERSP	LPPLSASHLQ	MRFFPDLAGT	DEHQFARGPP	650
hKv2.2	601	SSIDSFTSCA	TDFETETERSP	LPPLSASHLQ	MRFFPDLAGT	DEHQFARGPP	650
rKv2.2	601	SSIDSFTSCA	TDFETETERSP	LPPLSASHLQ	MRFFPDLAGT	DEHQFARGPP	650
cKv2.2	651	FLTLARGKGP	AGRDATLEYA	PVDITVSLDA	SGSKVGSHP	LOBSASESP	700
hKv2.2	651	FLTLARGKGP	AGRDATLEYA	PVDITVSLDA	SGSKVGSHP	LOBSASESP	700
rKv2.2	651	FLTLARGKGP	AGRDATLEYA	PVDITVSLDA	SGSKVGSHP	LOBSASESP	700
cKv2.2	701	KSSLKGSNPL	KRSRLKVNFK	ENRGSAPQTP	PSTARPLPVT	TADFSLTAPQ	750
hKv2.2	701	KSSLKGSNPL	KRSRLKVNFK	ENRGSAPQTP	PSTARPLPVT	TADFSLTAPQ	750
rKv2.2	701	KSSLKGSNPL	KRSRLKVNFK	ENRGSAPQTP	PSTARPLPVT	TADFSLTAPQ	750
cKv2.2	751	LITSTILLEET	PSQGDRCWA	PRARHTVRDL	PRGCPGFP	RNSLSHOER	800
hKv2.2	751	LITSTILLEET	PSQGDRCWA	PRARHTVRDL	PRGCPGFP	RNSLSHOER	800
rKv2.2	751	LITSTILLEET	PSQGDRCWA	PRARHTVRDL	PRGCPGFP	RNSLSHOER	800
cKv2.2	801	GGASLK....	850
hKv2.2	801	GGASLK....	850
rKv2.2	801	GGASLK....	850

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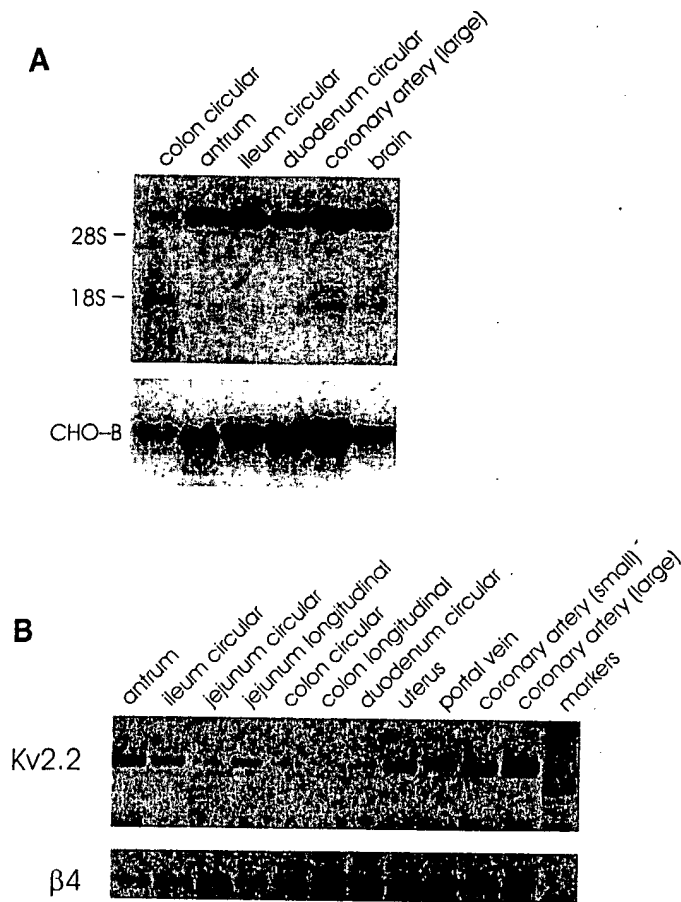


Fig. 2. A: Northern blot hybridized to Kv2.2-specific probe. Positions of 28S and 18S ribosomal RNA bands are indicated. RNA was prepared from several GI tissues and coronary artery as a representative vascular smooth muscle. CHO, Chinese hamster ovary. B: agarose gel depicting Kv2.2 and Kvβ₄-specific RT-PCR products generated from RNA derived from isolated smooth muscle cells. A Kv2.2-specific 823-bp amplification product was generated with specific primers from all smooth muscles tested. Whereas colonic longitudinal muscle displayed relatively little product, a longer exposure indicates the presence of a band at the appropriate molecular weight (data not shown). For Kvβ₄, a 724-bp amplification product was generated from all tissues examined. The markers are a 100-bp ladder with the lower molecular weight, high-intensity band being 600 bp and lighter bands increasing in size at 100-bp intervals.

levels of transcriptional expression in different smooth muscle cells.

Kv2.2 whole cell current in *Xenopus* oocytes. Oocytes injected with cRNA encoding hKv2.2 had large outward currents activated by depolarizations to potentials positive to -20 mV. There were no differences in the currents recorded from either cKv2.2 or hKv2.2. These currents were observed in >95% of oocytes injected with cRNA but not in water-injected controls. Furthermore, they were sensitive to K⁺ channel blockers and mediated by K⁺-selective single channels (see below).

Accordingly, we attribute them to the expression of injected Kv2.2 cRNA. Figure 3 summarizes the activation and steady-state inactivation properties of this current. Currents were elicited by 400-ms step depolarizations from -80 mV (Fig. 3A). A voltage-dependent conductance was activated at potentials positive to -20 mV. The midpoint of the conductance vs. voltage relationship was $+5 \pm 6$ mV ($n = 9$), and the conductance increased e -fold per 12 ± 1.1 mV ($n = 9$). There was no inactivation apparent during 400-ms depolarizations, and the current-voltage relationship was constructed by averaging the currents from eight oocytes and plotting the current at 400 ms as a function of step potential (Fig. 3B).

The kinetics of activation of smooth muscle Kv2.2 channels resembled those of the rat brain Kv2.2 channel (16) in that there was a distinct delay before the conductance increased and the rate of activation was slow compared with Kv1 channels. We characterized the rate of activation by measuring the time to half maximum current ($t_{1/2}$); $t_{1/2}$ decreased as the test potential was made more positive over the range -10 to +50 mV. Furthermore, at a test potential of +10 mV, $t_{1/2}$ from a holding potential of -80 mV was 97 ± 8.6 ms ($n = 14$).

Although there was no apparent inactivation over 400 ms, the Kv2.2 channels did inactivate during longer depolarizations. We measured the voltage-dependent inactivation of the channels using 20-s conditioning pulses to various potentials followed by 400-ms test steps to +20 mV (Fig. 3C, inset). Inspection of the currents during the conditioning steps revealed that a very slow inactivation process was still under way at the end of 20 s. Thus our results only approximate the true steady-state inactivation. We found that inactivation at the end of 20 s was well described by a Boltzmann function

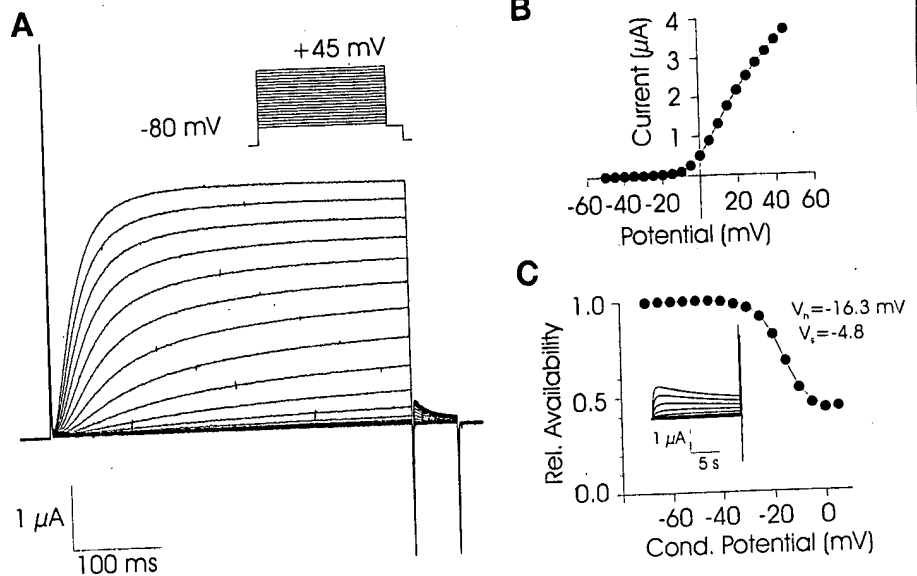
$$I = \frac{1 - C_{\min}}{1 + \exp \frac{(V - V_h)}{V_s}} + C_{\min}$$

where $C_{\min} = 0.42$ is the fraction of current that does not inactivate in 20 s, $V_h = -16.3$ mV is the prepulse potential for half inactivation, and $V_s = -4.8$ is the slope factor.

Pak et al. (24) found that the Kv2.1 channels encoded by *Drosophila* and mouse genes had different rates of recovery from inactivation so that *Drosophila* channels were nearly completely recovered after 1 s at -90 mV, whereas mouse channels took longer than 10 s to recover. We investigated this process in smooth muscle Kv2.2 channels using a similar protocol. In particular, oocytes were held at -60 mV and stepped for 10 s to +20 mV, for 1 s to -60 mV, and then to +20 mV for a 1-s

Fig. 1. Amino acid sequence of canine and human homologues of Kv2.2 (cKv2.2 and hKv2.2, respectively). Sequences are compared with rat Kv2.2 (rKv2.2) (16), and divergent amino acids are highlighted. Putative transmembrane spanning segments are indicated by solid bars above the sequence with the H5-pore-lining region shown. ●, Serines within consensus protein kinase A phosphorylation sites. ▼, Consensus tyrosine phosphorylation site. *, Putative N-linked glycosylation site. Most species divergence occurs in the carboxy-terminal region of the protein.

Fig. 3. Voltage-dependent activation and inactivation of Kv2.2 currents expressed in *Xenopus* oocytes. **A**: a family of currents elicited by 400-ms depolarizations from a holding potential of -80 mV (*inset*). **B**: current-voltage relationship obtained by measuring the currents at the ends of the steps in **A** plotted as a function of step potential. **C**: the characterization of voltage-dependent inactivation in another oocyte. The test current normalized to the current elicited after the -80 -mV conditioning step is plotted as a function of the conditioning potential. *Inset*: currents during 20-s conditioning pulses followed by test currents. V_h , prepulse potential for half inactivation. V_s , slope factor.



test pulse. In seven oocytes tested with this protocol the current inactivated at the end of the conditioning pulse to 0.59 ± 0.02 of the peak current and the current in the test pulse was 0.72 ± 0.03 of the peak current (Fig. 4A). That is, similar to mammalian Kv2.1 currents, smooth muscle Kv2.2 currents showed little recovery from inactivation during a 1-s repolarization to -60 mV. We investigated the time course of this process in more detail by increasing the duration of the interval between the conditioning and test steps up to 10 s (Fig. 4B). Test steps after recovery intervals <2 -s long elicited peak currents only slightly larger than the current at the end of the conditioning step, suggesting that Kv2.2 channels show "cumulative inactivation" (1, 24). For longer periods, recovery showed at least two phases, including a relatively rapid phase during which about one-half of the inactivation was removed. Full recovery took longer than 2 min.

Single-channel recordings from Kv2.2 expressed in oocytes. We recorded single-channel currents from three inside-out patches obtained from oocytes after mechanical removal of the vitelline membrane. Small conductance channels were observed in patches from oocytes

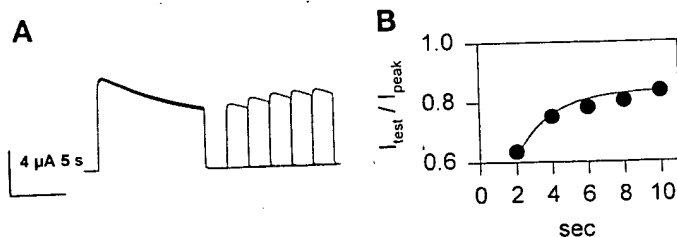


Fig. 4. Time course of recovery from inactivation. **A**: 5 superimposed traces during which the membrane potential was stepped from -80 to $+20$ mV for 10 s followed by recovery intervals of 2, 4, 6, 8, and 10 s before a 2-s test step to $+20$ mV. Runs were separated by 2-min rest periods to provide full recovery. **B**: the maximum current during the test step (I_{test}) divided by the peak current in the conditioning step (I_{peak}) as a function of the recovery interval (\bullet). The solid line has a time constant of 2 s and accounts for $\sim 60\%$ of the recovery from inactivation.

that had been kept for 3–10 days after injection with 50 ng of cRNA. Channel openings were rarely seen at potentials negative to -40 mV, but the likelihood of observing activity increased with further depolarization. Currents were recorded under the condition of symmetrical K^+ gradient, and we have selected the traces to show channel activity (Fig. 5A). In this case, the current-voltage relationship was linear (see Fig. 5B), reversed at 0 mV, and had a slope conductance of 15.3 ± 0.1 pS. Under an asymmetrical K^+ gradient (170 mM bath/intracellular vs. 2 mM pipette/extracellular), the channel current rectified in the outward direction. We fit the currents with the Goldman-Hodgkin-Katz current equation and obtained similar permeabilities (2.1×10^{-14} cm/s in symmetrical K^+ vs. 2.8×10^{-14} cm/s in asymmetrical K^+). Furthermore, the extrapolated reversal potential in the asymmetrical condition was close to the K^+ equilibrium potential, indicating that the channel was highly selective for K^+ .

Pharmacological characterization of Kv2.2 expressed in oocytes. In view of the differing pharmacological profiles of $I_{dK(s)}$ and $I_{dK(f)}$, it was of interest to characterize the response of the Kv2.2 currents to 4-AP and TEA. Figure 6, A and B, shows membrane currents elicited by steps from -60 to $+50$ mV in control and in the presence of 0.1 and 1 mM concentrations of 4-AP and TEA. Inhibition of Kv2.2 currents by 4-AP and TEA was dose dependent (Fig. 6, D and E). Furthermore, block by 4-AP was voltage dependent over the range $+10$ to $+50$ mV, being less effective at more positive potentials as has been demonstrated previously for Kv1.2 (14). At $+10$ mV, the IC_{50} was 1.5 ± 0.1 mM ($n = 10$) for 4-AP and 2.6 ± 0.9 mM ($n = 5$) for TEA. In addition we tested the effect of quinine, a known blocker of delayed rectifier current. We previously found that quinine blocked Kv1.5 ($IC_{50} = 365 \mu M$) (22). As shown in Fig. 6, C and F, quinine was a potent inhibitor of Kv2.2 with an IC_{50} of $14 \mu M$ ($n = 10$). Block by quinine was not voltage dependent. Kv2.2 was not blocked by charybdotoxin (>300 nM) or iberiotoxin (>300 nM).

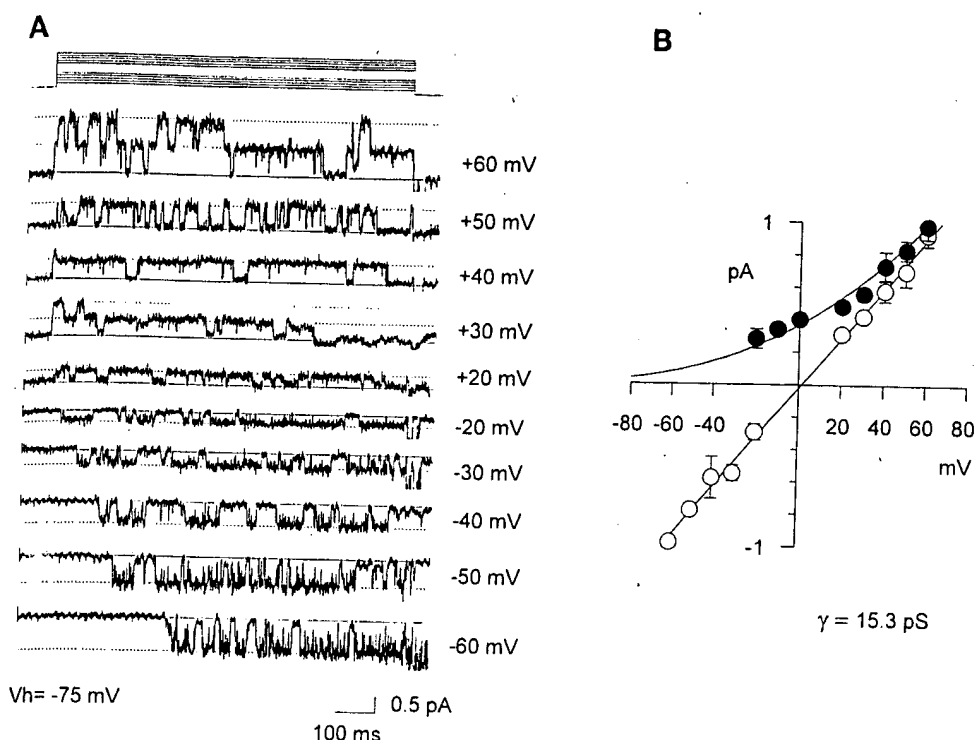


Fig. 5. Single-channel currents recorded from inside-out patches. A: records obtained under the condition of a symmetrical K^+ gradient (sweeps showing channel activity were selected). B: current-voltage relations in symmetrical (\circ) and asymmetrical (\bullet) K^+ gradients.

Delayed rectifier currents recorded from native myocytes. Because of the potent inhibition of Kv2.2 with quinine, we examined the sensitivity of the delayed rectifier current in native colonic myocytes to quinine in combination with 4-AP and TEA. 4-AP (5 mM) significantly increased the $t_{1/2}$ (23.5 ± 2.4 ms, $n = 6$) of the delayed rectifier current compared with control value (12.3 ± 1.6 ms) at +20 mV. However, TEA (10 mM) and quinine (1 μ M) did not change $t_{1/2}$ [11.5 ± 1.2 vs. 12.3 ± 1.0 ms, TEA ($n = 5$) vs. quinine ($n = 6$), respectively] (Fig. 7, A, B, and C). 4-AP reduced the delayed rectifier K^+ current [$35.1 \pm 2.9\%$, $n = 6$, 413 ± 17 pA (control) vs. 286 ± 14 pA (4-AP)]. Application of quinine (10 μ M) resulted in additional reduction of the current ($56.5 \pm 4.7\%$, $n = 6$, 179 ± 23 pA) in the presence of 4-AP (5 mM) (Fig. 7, D and F). However, application of quinine did not further reduce delayed rectifier current in the presence of TEA (10 mM) ($57.1 \pm 6.4\%$ of inhibition, $n = 7$; 173 ± 28 pA) and did not show additional reduction before and after treatment of TEA (10 mM, $55.3 \pm 5.1\%$ of inhibition, $n = 7$; 171 ± 22 vs. 376 ± 41 pA of control) (Fig. 7, E and F). These data are consistent with quinine, at low concentration (10 μ M), blocking the TEA-sensitive component of delayed rectifier current and blocking a slower activating current component.

DISCUSSION

Although the general importance of the plasmalemmal K^+ conductance to smooth muscle function is well accepted, the specific roles of individual K^+ channels are unclear and considerable effort is currently directed to the linking of K^+ channels to function in a variety of smooth muscles. One approach to this problem is based on the characterization of the components of K^+ current

in smooth muscle myocytes and the molecular identification of the channels that mediate them. In the present study, we have identified a molecular component of the delayed rectifier current in GI smooth muscle cells, determined its transcriptional expression in several GI muscle types, and studied the properties of the channels expressed by *Xenopus* oocytes.

Because the goal of our study was the identification of K^+ channels in smooth muscle myocytes, it was important to consider that smooth muscle tissues contain several cell types that might contribute mRNA to the PCR reaction used to detect expression of the K^+ channel gene. Accordingly, we analyzed the distribution of Kv2.2 transcriptional expression by Northern analysis and collected and amplified mRNA from myocytes prepared using methods developed for electrophysiological studies (4, 21, 25, 34). Thus we know that the Kv2.2 expression described here is at levels sufficient to be detected by Northern hybridization and that Kv2.2 expression is localized to the smooth muscle myocytes. Interestingly, we found Kv2.2 is expressed in GI, vascular, and uterine smooth muscle myocytes, i.e., in all types examined. We conclude that, similar to Kv1.5 (22), Kv2.2 is ubiquitously expressed in smooth muscles. We note that we tested myocyte preparations for the expression of Kv2.1 using primers designed to distinguish between Kv2.1 and Kv2.2 but failed to find evidence for the expression of Kv2.1 mRNA (data not shown). The expression of other, as yet unidentified, Kv2 family members in these cells is not excluded by this result. Recently, an auxiliary β -subunit (β_4) has been identified that couples specifically to Kv2.2 and enhances its expression level in *Xenopus* oocytes (9). Using primers specific for this cDNA, we have demonstrated the transcriptional expression of β_4 in GI and

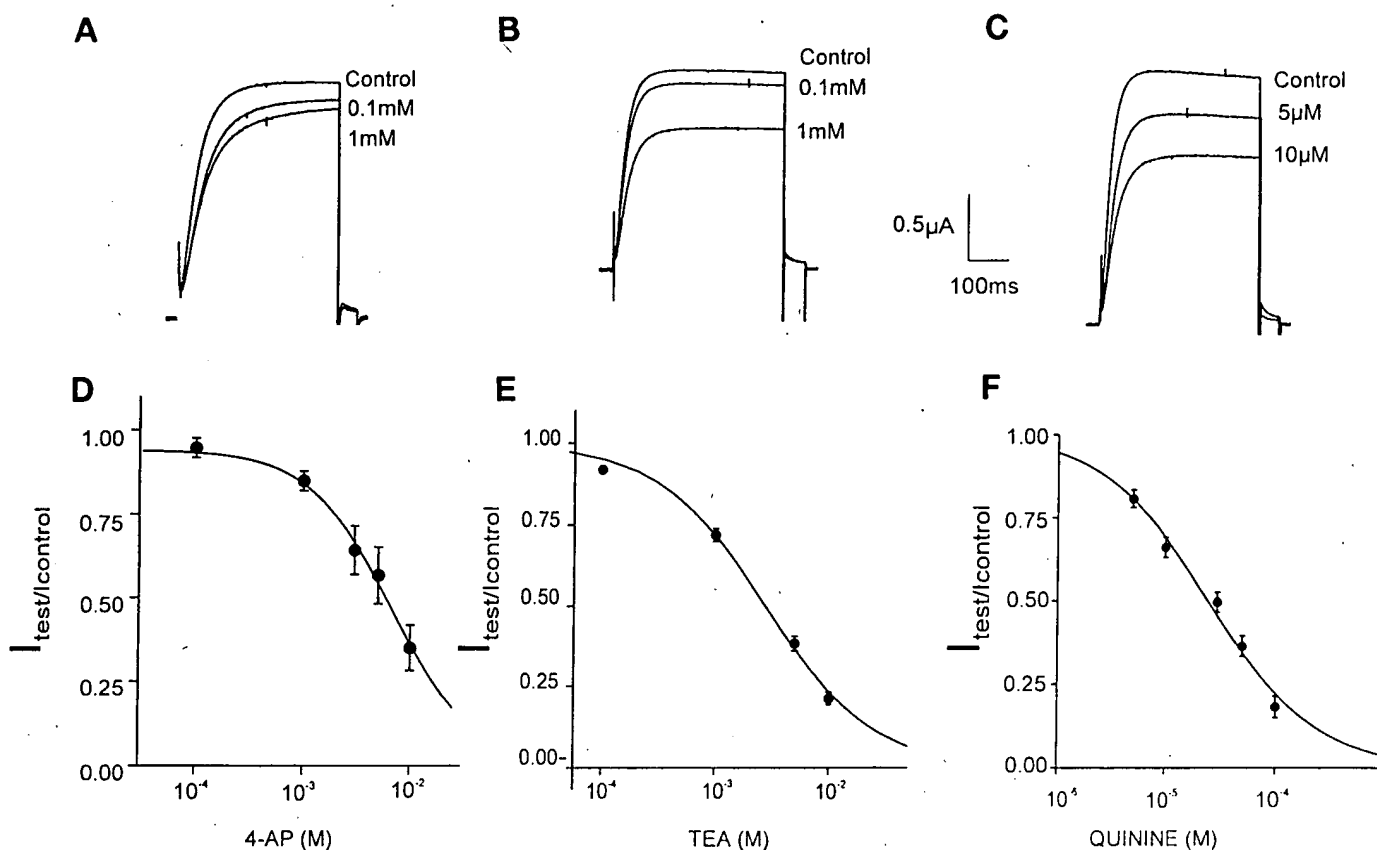


Fig. 6. Inhibition of Kv2.2 currents by 4-aminopyridine (4-AP) (A and D), tetraethylammonium (TEA) (B and E), and quinine (C and F). A–C: membrane currents elicited by steps from -60 to $+50$ mV in control and in the presence of the test compounds. D–F: average dose-response curves where each point is the averaged $I_{\text{test}}/I_{\text{control}}$, and error bars are means \pm SE. For 4-AP (D) $n = 10$, for TEA (E) $n = 5$, and for quinine (F) $n = 10$. IC_{50} values were obtained by fitting the data with the equation $I_{\text{test}}/I_{\text{control}} = [1 + ([\text{test compound}]/IC_{50})^n]^{-1}$ where $n \gg 1$. The smooth lines are the fits.

vascular smooth muscles. However, expression of Kv8.1, a new Kv channel with interesting regulatory properties toward the Kv2 family (15, 29), has not been detected in canine smooth muscles.

We find that Kv2.2 channels are activated at potentials positive to about -20 mV as are Kv2.2 channels from rat brain (16) and Kv2.1 channels (11, 31). This behavior is somewhat different from the *mShab* (Kv2.1) channels that are activated at more negative potentials (23). For all of these channels, the rate of activation is slow compared with that of members of the Kv1 family. In particular, we reported that smooth muscle cKv1.2 and cKv1.5 channels have $t_{1/2}$ values of 7.6 and 5.5 ms, respectively (14, 22), similar to other Kv1 channels. In contrast, the smooth muscle Kv2.2 channel described here has a $t_{1/2}$ of 97 ms (at $+10$ mV). This is similar to the behavior of Kv2.1 channels found by Frech et al. (11), faster than the channels described by Pak et al. (24), but slower than the activation of rat brain Kv2.2 (16). We also studied the single channels encoded by the smooth muscle Kv2.2 and found that they were selective for K^+ , were opened by depolarization, and that they had a single-channel conductance of 15.3 pS. This is similar to that reported by Fink et al. (9) for the Kv2.2 clone characterized by Hwang et al. (16) and similar to Kv1.2 and Kv1.5 smooth muscle K^+ chan-

nels, which have single-channel conductances of 14 and 9.8 pS, respectively (14, 22), making these channels underlying the delayed rectifier current difficult to dissect in native cells at the single-channel level.

The development of voltage-dependent inactivation of smooth muscle Kv2.2 qualitatively resembled that found for Kv2.1 channels (11, 31) in that a large fraction of the current is inactivated over many seconds. Interestingly, we found that the recovery from inactivation was very slow and followed a complex time course. That is, about one-half of the inactivation was reversed over 10 s, whereas full recovery of the current took longer than 2 min. In contrast, Pak et al. (24) found that recovery from inactivation by *Drosophila* Kv2.1 (*fShab*) channels was very fast (time constant of 0.4 s) compared with mouse channels (*mShab*, time constant of 4.2 s). For both channels, recovery was well described by a single exponential process. Thus the recovery from inactivation by smooth muscle Kv2.2 channels is uniquely complex and slow.

Although we have not characterized the recovery from inactivation in detail, it appears to have properties of cumulative inactivation described and modeled by Aldrich (1). Our results imply that although there is little inactivation during a single depolarization of 1- to 5-s duration [i.e., during a single slow wave, (32)], it is

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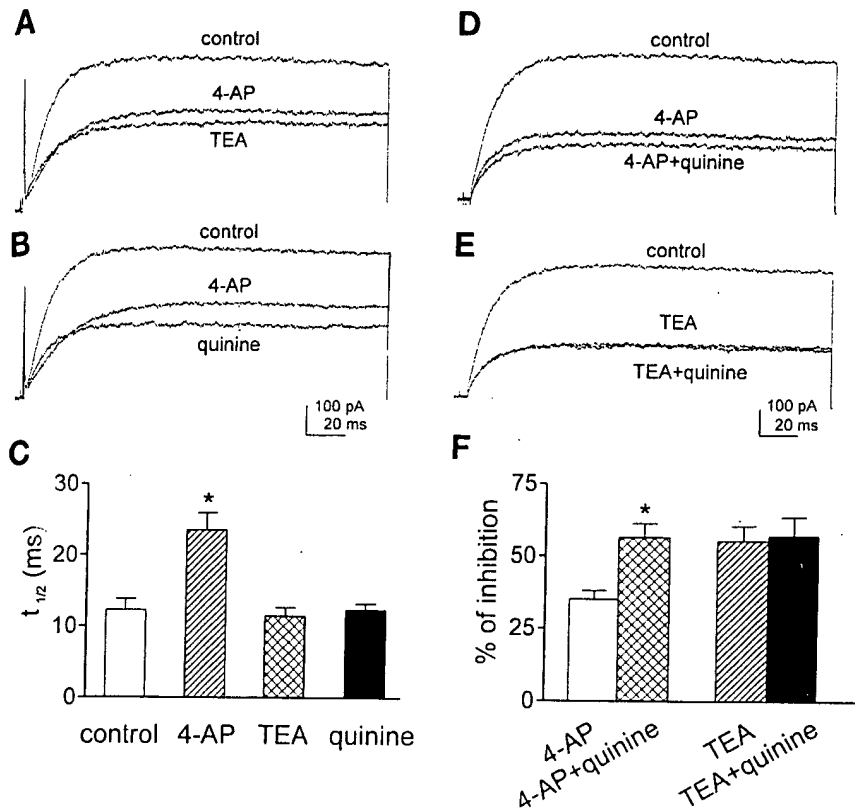


Fig. 7. Delayed rectifier currents were obtained with averaged currents from 10 episodes with step depolarization from a holding potential of -80 mV to test potential of $+20$ mV. Interepisode interval was 115 s. A and B: effects of 4-AP (5 mM, $n = 6$), TEA (10 mM, $n = 5$), and quinine (10 μ M, $n = 6$) in the same cell on the control delayed rectifier currents ($n = 17$). C: summary of time to half maximum current ($t_{1/2}$) of activation. 4-AP significantly increases $t_{1/2}$ of activation. $*P < 0.05$. D: current was elicited by step depolarization of $+20$ mV from a holding potential of -80 mV in the presence of 4-AP and additional application of quinine. E: obtained from the same protocol and patch as in D in the presence of TEA and TEA + quinine. F: summary of %current inhibition. $*P < 0.05$.

expected that inactivation will accumulate during rhythmic depolarizations separated by <10 s and that this inactivation will limit current through Kv2.2 channels in the steady state. An apparent continued development of inactivation during repolarization can be seen in the delayed rectifier currents of colonic smooth muscle myocytes [see Carl (4)].

Carl (4) used a pharmacological approach to identify the components of delayed rectifier K^+ current in canine circular colonic smooth muscle and concluded that there are three components of this current: $I_{dK(f)}$, $I_{dK(s)}$, and $I_{dK(n)}$. Carl (4) further reported that the current recorded in the presence of TEA [dominated by $I_{dK(f)}$] activated relatively quickly, whereas the current recorded in the presence of 4-AP [dominated by $I_{dK(s)}$] activated relatively slowly. The activation of smooth muscle Kv2.2 showed a sigmoidal time course as do other Kv1 and Kv2 currents. Although Carl (4) reported single exponential time constants to describe the activation of delayed rectifier currents in smooth muscle

myocytes, those currents also showed a sigmoidal activation with $t_{1/2}$ values at $+10$ mV of 20.6 ms in control [$I_{dK(f)} + I_{dK(s)}$], 30.3 ms in the presence of 4-AP [largely $I_{dK(s)}$], and 13.9 ms in the presence of TEA [largely $I_{dK(f)}$] (A. Carl, personal communication). Thus the rate of activation of $I_{dK(f)}$ is closer to that of the Kv1 channels, whereas the rate of activation of $I_{dK(s)}$ is closer to that of the Kv2 channels. Although there is an approximately threefold difference between $t_{1/2}$ values of $I_{dK(s)}$ and Kv2.2, quantitative comparison of these parameters is difficult. In particular, the pharmacological dissection of the native currents is likely to be incomplete and some of the current recorded in the presence of 10 mM 4-AP may have been contributed by Kv1 channels. Such a contribution might add to the difference in $t_{1/2}$ values seen here. Carl (4) found that the delayed rectifier current remaining in the presence of TEA [predominantly $I_{dK(f)}$] was quite sensitive to 4-AP ($IC_{50} = 23 \mu$ M). Thus the sensitivity of $I_{dK(f)}$ to 4-AP resembles that of the Kv1 currents [IC_{50} values $< 211 \mu$ M, (14, 23)], and

Table 1. Kinetic and pharmacological properties of cloned and native Kv channels from canine colonic smooth muscle

	$t_{1/2}$, ms	Activation Threshold, mV	Inactivation		IC_{50}			Channel Conductance, pS
			V_h	V_s	4-AP	Quinine	TEA _{out}	
cKv1.2	7.6	-40	-15 mV	7.7	75 μ M	250 μ M	<10 mM	14
cKv1.5	5.5	-40	-21 mV	7.0	211 μ M	365 μ M	<10 mM	9.8
$I_{dK(f)}$	7.8	-40	ND	ND	69 μ M	ND	<10 mM	20
cKv2.2	97	-20	-16.3 mV	4.8	1.5 mM	14 μ M	2.6 mM	15.3
$I_{dK(s)}$	31	-20	ND	ND	>1.5 mM	ND	2.2 mM	ND

4-AP, 4-aminopyridine. TEA, tetraethylammonium. $t_{1/2}$, Time to half maximum current. $V_h = V_{1/2}$, the prepulse voltage at which half the current inactivates. V_s , slope factor. $I_{dK(f)}$, fast activating K^+ current. $I_{dK(s)}$, slow activating K^+ current. ND, not determined.

this is one piece of evidence suggesting that $I_{dK(f)}$ is mediated by a heterotetramer formed of Kv1.2 and Kv1.5 subunits (27). The low sensitivity of $I_{dK(s)}$ to 4-AP is closer to the property of smooth muscle Kv2.2 (IC_{50} at +20 mV = 2 mM), suggesting that Kv2.2 may be a component of $I_{dK(s)}$. The Kv2.2 channels from smooth muscle are very sensitive to quinine [IC_{50} = 14 μ M compared with 365 μ M for Kv1.5 (22)]. Therefore, we performed experiments on native colonic myocytes to examine if quinine at low concentrations would preferentially block $I_{dK(s)}$. Figure 7, A–C, shows that in the presence of 5 mM 4-AP [a concentration that blocks all of $I_{dK(f)}$ but only a portion of $I_{dK(s)}$], the overall delayed rectifier current is reduced and the $t_{1/2}$ of the remaining current is significantly increased. Combining TEA (5 mM) or quinine (10 μ M) [agents that should only block $I_{dK(s)}$ at these concentrations] with 4-AP further reduces the current and changes the $t_{1/2}$ back to the original level in the absence of blockers. The converse experiment in Fig. 7E shows that when TEA is applied, which only blocks $I_{dK(s)}$, the addition of 10 μ M quinine does not further reduce the current and does not change the activation rate. A comparison of the kinetic and pharmacological properties of cloned and native Kv channels from canine colonic smooth muscle is shown in Table 1.

In summary, the results reported here and in our previous study (27) suggest that Kv1.2 and Kv1.5 underlie $I_{dK(f)}$, whereas Kv2.2 may be a component of $I_{dK(s)}$ based on similarities of the voltage dependencies of activation, activation kinetics, and the pharmacology of K^+ currents recorded in smooth muscle myocytes and in *Xenopus* oocytes. However, several issues remain outstanding. First among these is the difficulty comparing currents encoded by mammalian genes expressed in *Xenopus* oocytes with those recorded in native cells. In addition, the potential functions of accessory subunits or Kv channels not yet identified at the molecular level must be considered. Definitive assignments must await antibody studies at the immunocytochemical level combined with antisense knockout experiments in smooth muscle myocytes.

Beech and Bolton (2), Okabe et al. (21), and Robertson and Nelson and colleagues (25, 26) have reported delayed rectifier currents in single smooth muscle cells, outside the GI tract, that are relatively insensitive to 4-AP. Although direct comparisons are not possible because of the voltage sensitivity of 4-AP inhibition of K^+ currents (6, 28) and the examination of 4-AP block at different step potentials, our finding Kv2.2 mRNA in all smooth muscle myocytes examined (both GI and non-GI) raises the possibility that this clone may represent a component of the slowly activating K^+ current of smooth muscle myocytes from visceral and vascular smooth muscles.

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The nucleotide sequences for hKv2.2 and cKv2.2 have been submitted to the GenBank database with accession nos. U69962 and U69963, respectively.

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